

## CORAL PURIFICATION METHOD, AND CORAL THUS OBTAINED

The present invention relates to a method of purifying coral, and to the coral obtained by said method.

5 The invention is of particular application in the medical field.

Coral, which has a porous structure, is essentially (about 97%) composed of mineral material, mainly calcium carbonate, with the remainder (about 3%) being  
10 constituted by oxides of magnesium and iron together with organic substances which are responsible for its color. Its hardness varies from 2.5 to 3.5 on the Mohs scale.

The constituent calcium carbonate of coral has a metastable aragonite type crystallographic structure  
15 which can easily be transformed into calcite, especially under the effect of a temperature of about 260°C.

The organic matter in coral contains a proteinaceous material, but it is substantially free of lipid material.

Several bone substitutes based on coral currently  
20 exist and have been approved by the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) [French Health Safety Products Agency]. One of those products, sold by INOTEB under the trade name BIOCORAL®, is obtained by treating the coral at the surface of its  
25 pores, which can eliminate a portion of the organic substances mentioned above.

A more thorough morphological analysis of coral (thermal scanning electron microscope coupled with mass spectroscopy) shows, however, that said organic  
30 substances are present not only in the pores of the coral, but also at the grain boundaries of the calcium carbonate particles. The treatment carried out for the BIOCORAL® product cannot extract organic substances located at the grain boundaries, which represents about  
35 90% of the total organic substances.

One problem which the present invention aims to overcome is thus to provide coral that is substantially

free of organic matter, in particular proteins, and that can be used to fabricate bone substitutes with a quality comparable to that of existing synthetic implants.

5 The term "coral substantially free of organic matter" as used in the present description means coral containing not more than half as much organic substance as untreated coral.

A further problem which the present invention aims to overcome is to provide a method which can eliminate  
10 organic substances present in the coral without modifying its crystalline structure.

It has now been discovered, and this forms the basis of the invention, that treating coral with a fluid under supercritical conditions in an optimized temperature and  
15 pressure range can overcome the problems mentioned above, which problems are not overcome with known prior art methods.

Hence, in a first aspect, the invention provides a method of extracting organic substances present in coral,  
20 which method consists in treating the coral with a fluid or a mixture of fluids in the supercritical state without modifying the crystalline structure of said coral, at a temperature of less than 270°C, preferably 260°C or less, and more preferably 250°C or less, and at a pressure  
25 which is much higher than the critical pressure of said fluid or mixture of fluids, for example of the order of at least 3 times, and preferably at least 5 times said critical pressure.

The use of a fluid in a supercritical state to  
30 extract organic substances has been described in European patent document EP-A-0 603 920. However, that document relates only to the treatment of a bone tissue and uses carbon dioxide to extract lipids, i.e. substances which are not found in coral. According to that document, in  
35 order to extract proteins, a complementary chemical or enzymatic treatment is employed that makes use of additional compounds such as detergents or oxidizing

agents, which would alter the structure of the coral. Further, in that document, the treatment is carried out at a pressure close to the critical pressure of carbon dioxide.

5        In the method of the invention, a fluid is used under conditions that are termed "supercritical", i.e. the fluid has the invasive properties of a gas while behaving like a liquid. For this reason, the fluid can diffuse into the porous structure of the coral without  
10 any wettability problems, which can optimize extraction of the organic substances present in the coral, in particular proteins (which are also termed below the "organic matrix").

15        The fluid or the mixture of fluids suitable for use in the method of the invention must have a critical temperature of less than 270°C, preferably 260°C or less, and more preferably 250°C or less, and must dissolve organic matter.

20        Examples which may be mentioned are ethanol, acetone, or a mixture of one of those two fluids with carbon dioxide and having a critical temperature of less than 270°C. Advantageously, ethanol is used because of its availability and its inexpensive and non toxic nature. The critical temperature of ethanol is 240°C and  
25 its critical pressure is 6.12 MPa [megapascals].

      When the fluid is ethanol or acetone, the treatment temperature for the coral is advantageously in the range 240°C to 260°C.

30        When a mixture of fluids is used such as an ethanol-carbon dioxide mixture, the coral treatment temperature is advantageously in the range 80°C to 100°C.

      The method of the invention is carried out at a sufficient pressure which is much higher than the critical pressure of the fluid or mixture of fluids used  
35 to prevent transformation of the crystalline structure of the coral. In this regard, the critical pressure of the fluids mentioned above is easily achieved.

With ethanol or acetone, the coral treatment pressure is advantageously in the range 300 MPa to 450 MPa, preferably in the range 350 MPa to 450 MPa.

5 With a mixture of fluids such as an ethanol and carbon dioxide mixture, the coral treatment pressure is advantageously in the range 30 MPa to 50 MPa, preferably of the order of 40 MPa.

The treatment period is not critical per se and generally varies between a few minutes and a few hours  
10 depending on the fluid used. In the case of ethanol or acetone, this period is advantageously in the range 15 min [minutes] to 240 min, and preferably about 1 h [hour].

Given the mineral chemical composition of coral, the  
15 fluid or mixture of fluids of the invention is advantageously used without any additional compound such as a detergent or oxidizing agent which could alter the structure of the coral.

In a second aspect, the invention concerns coral  
20 substantially free of organic substances, as obtained by the method described above.

In a third aspect, the invention provides bone substitutes produced from coral substantially free of organic substances. Said bone substitutes may be  
25 obtained using methods that are well known to the skilled person.

The invention is illustrated below using the following examples.

**EXAMPLE 1: Supercritical treatment of coral**

30 Untreated coral was treated in a high water pressure generating device in a chamber with a fixed volume. This chamber, produced from a high strength alloy, was 229 mm [millimeters] deep and had an inside diameter of 55 mm. It was closed by a mushroom-shaped closure supporting the  
35 metallic seal which provided a seal by deformation thereof. The seal on start-up (without the autoclave being pressurized) was produced by raising the closure

using 8 screws in the upper portion. The pressure applied to the chamber by the pump or the temperature compress the seal and thus seal the system.

5 The temperature was measured inside the chamber using a thermocouple mounted in the upper portion of the reactor. It was in direct contact with the pressurized fluid.

The coral was placed in flexible 20 mL [milliliter] Teflon PFA bottles (ref M77400, Fisher Bioblock Scientific) which can resist temperatures of close to 10 300°C and tolerate deformation due to pressure. Equal pressures were exerted on the inside and outside walls of the bottle. Since the bottle contained very little or no air, the liquid present inside the bottle and the 15 transmitting liquid (water) were at the same pressure, and thus the wall was only deformed slightly due to the compressibility of water. The treatment was carried out in the presence of ethanol as the "supercritical" fluid at a temperature of 240°C to 260°C, preferably of the 20 order of 250°C, and at a pressure of 300 MPa to 400 MPa, preferably of the order of 350 MPa for 15 min to 240 min, preferably for about 1 h.

**EXAMPLE 2: Determination of the quantity of residual organic substances in the treated coral**

25 The sample obtained in Example 1 was ground with a mortar and weighed (UM mass - untreated matter - about 3 g [grams]). 3 g of the powder obtained was dissolved in 135 mL of 0.1N HCl with stirring for 1 h. The solution obtained thereby was dialyzed through a membrane with a 30 molecular weight of 12000 to 16000 (reference MCWO) then frozen at -80°C and freeze dried. The freeze dried material was then weighed (OM mass - organic matter - about 3 g.

For comparison purposes, the same experiment was 35 carried out on an untreated coral sample (aragonite) and on a sample of BIOCORAL®.

Figure 1 shows the ratio OM/UM for each of the three samples. It can be seen that the "supercritical" treatment of the invention can produce a coral containing about half the organic substances as the BIOCORAL® product.

**EXAMPLE 3: Determination of the quantity of residual proteins in treated coral**

*1/ Preparation of working solution and samples:*

- 50 volumes of solution A was mixed with 1 volume of solution B. The working solution was slightly cloudy to start with, then cleared rapidly with gentle vortex stirring. It was an opalescent green color;
- the samples were ground with a mortar and the powder obtained was weighed;
- 1 g of powder was dissolved in 45 mL of 0.6N HCl over 1 hour, with stirring.

*2/ Procedure for determination:*

- the water-soluble fraction was assayed in water: 1.2 mg [milligrams] of organic matter was dissolved in 75  $\mu$ L [microliters] water;
- the residue following centrifuging was dissolved in 75  $\mu$ L of 0.3N NaOH;
- 25  $\mu$ L of each standard (0  $\mu$ g/mL, 5  $\mu$ g/mL, 25  $\mu$ g/mL, 50  $\mu$ g/mL and 250  $\mu$ g/mL [micrograms/milliliter]) of each sample (prepared in the same manner as the assay samples) and they were distributed into wells of a 96-well plate;
- the plate was covered and incubated for 30 min at 37°C;
- the optical density at 562 nm [nanometers] was read once the plate had cooled to ambient temperature.

For comparison, the same experiment was carried out on a sample of raw coral (aragonite) and on a sample of BIOCORAL®.

Figure 2 shows the protein/UM ratio for each of the three samples. It can be seen that the "supercritical" treatment of the invention can produce a coral from which

about twice as much protein has been extracted compared with the BIOCORAL® product.

**EXAMPLE 4: Demonstrating cell proliferation of bone cells on treated coral**

5       Cylinders (diameter = 12 mm) were cut into 1 mm thick disks using a diamond saw then sterilized by dry heat at 100°C for 1h30 (3 cycles). The disks were deposited on a bed of 0.1% agarose in culture dishes (NUNC; 24 wells, 2 cm<sup>2</sup> [square centimeters]). The cell  
10       suspension was deposited onto each disk in an amount of 20000 MG63 cells/cm<sup>2</sup> (1.13 cm<sup>2</sup> x 2, since porosity - 22600 cells, i.e. about 50000 cells per sample), in a volume of 100 µL of complete culture medium (IMDM: Iscove's Modified Dubelcco's Medium, 10% FCS - fetal calf serum).  
15       After 30 min to allow the cells to adhere, it was supplemented with 1 mL of medium. The medium was changed every two days.

      The complete culture medium was removed and rinsed with medium alone. The cells were starved for 17 hours  
20       (medium alone + 10% FCS, tritiated thymidine, 10 µCi/mL [microcuries/milliliter] (ref: TRK 758 AMERSHAM), i.e. 10 µL of <sup>3</sup>HT/volume of complete medium). The supernatant was eliminated (retained for subsequent elimination) then the material was removed from the agarose and it was  
25       transferred to a plastic well. It was rinsed twice with PBS 1X (or Hank's 1X), then the cells were fixed with cold 100% methanol, for 10 min at 4°C. It was rinsed twice with PBS 1X (or Hank's 1X) and precipitated with cold 5% TCA for 20 minutes at 4°C. The supernatant was  
30       eliminated and it was rinsed four times with PBS 1X (or Hank's 1X).

      It was incubated in 0.3N NaOH for 2 h at ambient temperature (to dissolve the cell layer) without exceeding 300 µL to 500 µL of NaOH. The solution was  
35       recovered in a scintillation tube to which 5 mL of liquid scintillant had been added, and the radioactivity of the

solution and the material were determined using a  $\beta$  counter (negligible radioactivity).

For comparison, the same experiment was carried out on a sample of untreated coral (aragonite) and on a  
5 sample of BIOCORAL®.

The results are shown in Figure 3. It can be seen that cell proliferation of MG63 cells on the coral treated in accordance with the invention was identical to that observed with the BIOCORAL® product.

10 These results suggest that coral treated in accordance with the present invention may be used in the fabrication of bone substitutes.